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Effect of Vitamin A Supplementation on Immunoglobulin G Subclass Responses to Tetanus Toxoid in Children

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Previously, we demonstrated that administering vitamin A supplements to children resulted in a significant increase in the immunoglobulin G (IgG) response generated against a vaccine dose of tetanus toxoid (TT) (R. D. Semba et al., J. Nutr. 122:101–107, 1991). However, from these analyses we could not determine whether there was an increase in levels of IgG of the subclass presumed to be important for protection against challenge by the toxin or whether there was simply a general increase in the levels of all of the IgG subclasses expressing anti-TT activity. The goal of this study was to determine the profile of the anti-TT IgG subclasses in children receiving vitamin A supplementation or a placebo in order to assess the potential utility of the enhanced anti-TT response. In a randomized, double-masked, placebo-controlled clinical trial, the levels of the different anti-TT IgG subclasses were measured in 139 Indonesian preschool children (3 to 6 years of age) 2 weeks before and 3 weeks after immunization. Baseline anti-TT levels and immunization histories were used to separate those children who were responding to TT for the first time from those who responded in a secondary fashion because of previous exposure to TT. Children who were given vitamin A prior to immunization had significant increases in IgG1 levels regardless of whether they were undergoing primary or memory reactions. In the group of individuals who underwent a secondary response to TT, vitamin A supplementation was also associated with a modest but significant change in the levels of anti-TT IgG3. There were only minor changes in the levels of anti-TT IgG2 and IgG4. Since IgG1 is the subclass associated with a protective response to TT immunization, these results suggest that vitamin A supplementation may be a safe and effective intervention to enhance the relevant humoral response to TT and other vaccine antigens.

Experimental and clinical evidence has shown that vitamin A status has a significant influence on the ability of a host to resist infection and disease. Vitamin A deficiency is associated with a decreased resistance to infection and an increase in morbidity and/or mortality associated with infectious diseases (14, 20). The increased susceptibility to infectious agents and the heightened morbidity and mortality are quickly reversed by repletion with retinol. Vitamin A and other retinoids appear to exert powerful effects as adjuvants when used as supplements during immunization of vitamin A-replete animals and humans (4, 7, 9, 12, 19). Both humoral and cell-mediated immune responses appear to be enhanced by retinoids (2, 6, 10, 17, 18). The mechanism through which retinoids enhance immune responsiveness remains unclear.

Recently we demonstrated that vitamin A-deficient and clinically healthy children who received high-dose vitamin A had significantly enhanced total immunoglobulin G (IgG) responses to tetanus toxoid (TT) (16). However, from these results it was not clear whether vitamin A was potentiating the antigen-specific subclass typically associated with a protective immune response to TT in children or whether there was a general, nonspecific increase in levels of all anti-TT IgG subclasses. The issue of subclass expression is of importance because each of the four IgG subclasses produced by human B cells has specific biological properties that direct humoral immune responses along distinct functional pathways. In the context of protective immune responses induced by vaccination, certain functional pathways are more desirable than others. The increase in levels of IgG associated with vitamin A supplementation will have its greatest impact on host immunity only if the proper IgG subclasses are produced. The present study was conducted to gain insight into the IgG subclass responses to TT in children who received vitamin A supplementation.

MATERIALS AND METHODS

A randomized, double-masked, placebo-controlled clinical trial with 236 preschool children, aged 3 to 6, was conducted in Bandung, West Java, Indonesia. Children with mild vitamin A deficiency (i.e., mild xerophthalmia; $n = 118$) and clinically healthy children ($n = 118$) had a baseline blood drawing and were randomized to receive oral high-dose vitamin A (200,000 IU) or a placebo, creating four allocation groups. The children were seen two additional times, at 2 weeks, when they were vaccinated, and at 5 weeks, when blood was drawn for analysis of plasma vitamin A and anti-TT levels. Details of this clinical trial have been described elsewhere (16).

During the 5-week period of the study, the children were examined three times by a pediatrician and an ophthalmologist. Detailed anthropometry was carried out (23), and children with values below 80% of the National Center for Health Statistics median weight for height (11) were excluded from the study. Individuals with serious illness were also excluded from the study and provided with appropriate treatment. Baseline and follow-up plasma vitamin A and transthyretin levels were measured by high-performance liquid chromatog-

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TABLE 1. Characteristics of the four allocation groups

Group and supplement	No. of participants (female/male)	Age (mo)[a]	Wt for ht[a,b]	Ht for age[a,b]	Plasma vitamin A concn (μmol/liter)[a]		Concn of transthyretin (mg/liter)[a]
					Baseline	Follow-up	
Healthy							
Vitamin A	11/25	57.0 (1.8)	92.4 (0.9)	89.1 (0.7)	0.77 (0.05)	1.61 (0.08)	149 (4)
Placebo	11/20	58.0 (1.8)	96.2 (1.2)	90.4 (0.7)	0.75 (0.05)	0.75 (0.04)	151 (6)
Xerophthalmic							
Vitamin A	10/26	59.0 (1.8)	95.6 (1.0)	89.4 (0.8)	0.59 (0.04)	1.48 (0.07)	144 (4)
Placebo	12/24	56.8 (1.9)	97.4 (1.5)	89.7 (0.8)	0.55 (0.04)	0.62 (0.04)	140 (5)

[a] Values are means (values in parentheses are standard errors of the means).
[b] Expressed as percentages of the median of National Center for Health Statistics criteria (11, 23).

raphy (1) and radial immunodiffusion (Behring, Somerville, N.J.), respectively. Two weeks after administration of vitamin A or the placebo, all children were immunized with diphtheria-pertussis-tetanus vaccine (Lederle Laboratories, Pearl River, N.Y.). Three weeks after immunization, follow-up blood samples were obtained.

The study included children who had been immunized previously with TT as well as individuals who had never been immunized with tetanus antigen. Baseline titers of IgG to TT and immunization histories obtained from the mothers were two indicators which were used to separate primary from secondary responses to TT (16). In the analysis, the agreement of immunization histories and baseline anti-TT IgG titers (n = 139) appeared to be the most reliable criterion for distinguishing primary from secondary responses to tetanus. Thus, data from the 97 children whose histories and IgG titers were not concordant were excluded from this study in order to minimize misclassification of subjects as to their history of exposure to TT.

Baseline and follow-up IgG1, IgG2, IgG3, and IgG4 antibody responses to TT were analyzed by enzyme-linked immunosorbent assay (16). IgG subclass responses were measured by using a 1:2,000 dilution of mouse anti-IgG subclass monoclonal antibody (Sigma Chemical, St. Louis, Mo.) and then washing and incubating microtiter plates with a 1:1,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Sigma). Substrate development was carried out with o-phenylaminediamine, and A_{492}s were read. The relative amounts of each IgG subclass were measured by comparing the optical densities of the test sera with the optical densities obtained from standard quantities of purified human IgG of the appropriate subclasses (The Binding Site, Birmingham, United Kingdom) and were expressed as units per liter of blood.

The IgG subclass responses to TT were compared between vitamin A and placebo groups by using Student's two-tailed t tests, paired t tests, and multiple linear regression analysis.

All study procedures were approved by the Nutrition Research and Development Centre, Ministry of Health, Government of Indonesia, and by the Joint Committee on Clinical Investigation of the Johns Hopkins University School of Medicine and Johns Hopkins Hospital.

RESULTS

There were no significant baseline differences in age, sex, weight for height, height for age, or transthyretin levels among the four allocation groups (Table 1). Plasma transthyretin levels were used as a measure to assess the relative protein and energy status of the children in the study. At baseline, children without clinical signs of vitamin A deficiency who received vitamin A supplementation or the placebo had mean plasma vitamin A levels of 0.77 and 0.75 μmol/liter, respectively. As expected, children with xerophthalmia had mean plasma vitamin A levels of 0.59 μmol/liter (supplementation group) and 0.55 μmol/liter (placebo group), which were significantly lower than levels for the clinically healthy children ($P < 0.0004$). The overall vitamin A status of this population of children was near the conventional cutoff of 0.7 μmol/liter, which is frequently used to define adequate versus marginal vitamin A status (5, 16).

At 5 weeks after the administration of 200,000 IU of vitamin A, the clinically healthy and xerophthalmic groups had mean plasma vitamin A concentrations of 1.61 and 1.48 μmol/liter, respectively (Table 1). There was no significant change in the plasma vitamin A levels of the children who received the placebo. In the analysis of primary and secondary responses to TT, we grouped together the data for all the children who received vitamin A and compared their responses with the responses of individuals who received the placebo, regardless of baseline vitamin A status. These groupings were used because plasma vitamin A levels at follow-up were significantly different (by Tukey's test) only between children who received vitamin A and those who did not.

When the geometric mean differences (baseline versus 3 weeks postvaccination) of the concentrations of the four IgG subclasses were evaluated, IgG1 was found to have dominated both the primary and secondary immune responses to TT. Vitamin A supplementation was associated with a twofold enhancement in the anti-TT IgG1 response in children responding in a primary fashion (Fig. 1). IgG2, IgG3, and IgG4 contributed only slightly to the primary response, and vitamin A supplementation had no effect on the levels of synthesis of these IgG subclasses.

For children undergoing a secondary response to TT, vitamin A supplementation resulted in a nearly fourfold enhancement in the geometric mean IgG1 response to TT immunization (Fig. 2A). The memory anti-TT IgG3 response was also increased in the group given vitamin A (Fig. 2B). Changes in TT-specific IgG2 and IgG4 synthesis were not influenced by the vitamin A status of children undergoing a secondary response.

When the individual subclass responses to TT in the four allocation groups were assessed by using a multiple linear regression model to adjust for primary and secondary responses, vitamin A supplementation was associated with significant increases in levels of anti-TT IgG1 ($P < 0.03$) and IgG3 ($P < 0.04$) only.

An attempt to carry out a similar type of analysis on the humoral response to diphtheria toxin was made. In contrast to

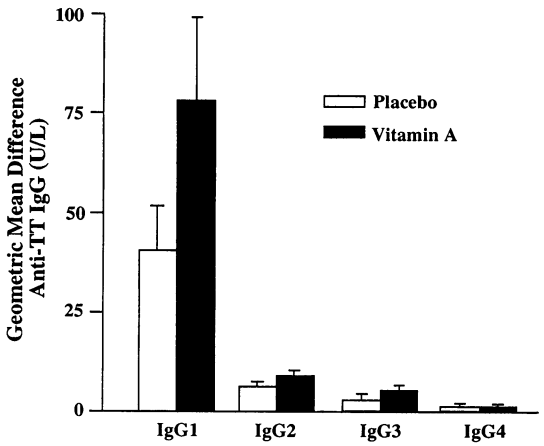

FIG. 1. Effect of vitamin A supplementation on the changes in the IgG subclass levels in children undergoing a primary immune response to TT. The values represent the geometric mean differences between the anti-TT IgG subclass levels at baseline (2 weeks prior to immunization) and the IgG subclass levels 3 weeks postimmunization. The optical densities of the test sera were converted to units per liter of serum through comparisons with values obtained from known amounts of IgG subclass standards. Each horizontal bar represents 1 standard error of the mean.

TT, we found that it was very difficult to employ immunization histories and baseline antigen-specific IgG levels to distinguish children who were undergoing a primary immune response from those who were mounting a secondary anti-diphtheria toxin response. The difficulty in distinguishing primary from secondary responses was probably due to the high levels of natural exposure to the diphtheria antigen experienced by children in this region (13). We did not measure the humoral responses mounted against pertussis antigens.

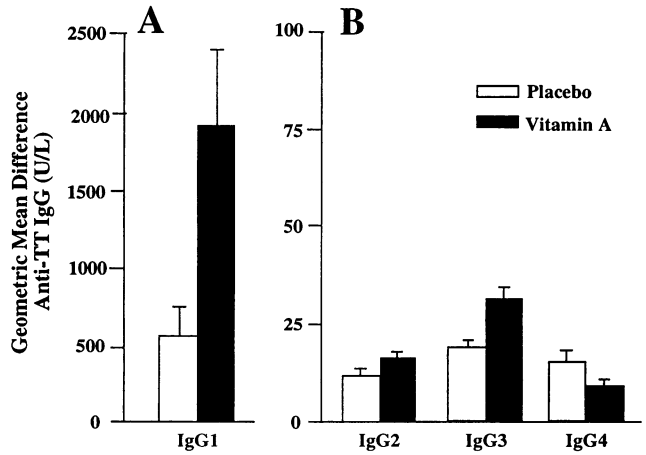

FIG. 2. Effect of vitamin A supplementation on the changes in IgG1 (A), IgG2, IgG3, and IgG4 (B) levels in children undergoing a secondary immune response to TT. The values represent the geometric mean differences between the anti-TT IgG subclass levels at baseline (2 weeks prior to immunization) and the IgG subclass levels 3 weeks postimmunization. The optical densities of the test sera were converted to units per liter of serum through comparisons with values obtained from known amounts of IgG subclass standards. Each horizontal bar represents 1 standard error of the mean.

DISCUSSION

The IgG1 subclass dominated both the primary and memory anti-TT responses in this group of 139 3- to 6-year-olds. Rubin et al. (15) and Stevens et al. (21) also showed that IgG1 is the most abundant IgG subclass in the anti-TT response of children. Also consistent with the results of our study, detectable levels of IgG3 and low-level IgG2 and IgG4 responses have been reported to be part of the profile of the humoral response to TT in children (15). In adults, although IgG1 remains the predominant anti-TT immunoglobulin, significant levels of IgG4 and trace amounts of IgG2 and IgG3 are produced (15, 21). The reason for this apparently age-related change in the subclass composition of the humoral response to TT is not clear but may be related to such factors as the maturity of the immune system and the history of antigen exposure.

Vitamin A supplementation selectively enhanced the production of anti-TT IgG1, the subclass most typically associated with an immune response capable of conferring protection against challenge with tetanus toxin (21). This increase in anti-TT IgG1 levels was observed regardless of the individual's initial vitamin A status. These results suggest that providing vitamin A 2 weeks prior to immunization is an effective way to heighten the protective immune response to natural challenge with tetanus toxin. It is not known whether oral vitamin A will act as an adjuvant when given simultaneously with immunization, although studies have demonstrated that other retinoids have an adjuvant effect when given at the time of immunization (9, 19).

The mechanism through which vitamin A selectively increases the level of the IgG1 subclass of anti-TT antibodies and causes little or no increase in the levels of the other three IgG subclasses is not clear. The enhanced production of a single subclass suggests that vitamin A supplementation is not affecting induction of immunoglobulin synthesis through a general stimulation pathway. Although it has been shown that vitamin A does have a direct effect on B cells and immunoglobulin synthesis (3, 22), it is possible that in our study vitamin A was acting indirectly on B cells by enhancing or modifying the function of T cells or antigen-presenting cells. With these same children, it was demonstrated that the vitamin A supplementation did have an effect on T cells, resulting in increased CD4/CD8 ratios and an increase in the proportion of naive $CD4^+$ T cells (17).

Recently the World Health Organization and the United Nations Children's Fund have proposed linking vitamin A supplementation with the Expanded Programme on Immunization (8, 24). The usual childhood immunization contacts of the Expanded Programme on Immunization involve diphtheria-pertussis-tetanus and oral polio vaccination at 6, 10, and 14 weeks of age. The results of the present study suggest that if oral vitamin A is given at these three immunization contacts, it may have the benefit of enhancing immune responses to vaccination. Further studies are in progress in Indonesia to determine whether linking vitamin A supplementation with the Expanded Programme on Immunization will enhance immune responses to immunization.

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